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Sheeppox Virus Kelch-Like Gene *SPPV-019* Affects Virus Virulence[†]

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Sheeppox virus (SPPV), a member of the *Capripoxvirus* genus of the *Poxviridae*, is the etiologic agent of a significant disease of sheep in the developing world. Genomic analysis of pathogenic and vaccine capripoxviruses identified genes with potential roles in virulence and host range, including three genes with similarity to kelch-like genes of other poxviruses and eukaryotes. Here, a mutant SPPV with a deletion in the *SPPV-019* kelch-like gene, Δ KLP, was derived from the pathogenic strain SPPV-SA. Δ KLP exhibited in vitro growth characteristics similar to those of SPPV-SA and revertant virus (RvKLP). Δ KLP-infected cells exhibited a reduction in Ca^{2+} -independent cell adhesion, suggesting that *SPPV-019* may modulate cellular adhesion. When inoculated in sheep by the intranasal or intradermal routes, Δ KLP was markedly attenuated, since all Δ KLP-infected lambs survived infection. In contrast, SPPV-SA and RvKLP induced mortality approaching 100%. Lambs inoculated with Δ KLP exhibited marked reduction or delay in fever response, gross lesions, viremia, and virus shedding compared to parental and revertant viruses. Together, these findings indicate that *SPPV-019* is a significant SPPV virulence determinant in sheep.

Sheeppox virus (SPPV) is the etiologic agent of a highly significant disease of sheep and the type species of the genus *Capripoxvirus*, one of the eight genera of the subfamily *Chordopoxvirinae* of the *Poxviridae* (44). Other capripoxviruses (CaPVs) include goatpox virus (GTPV) and lumpy skin disease virus (LSDV), which cause disease in goats and cattle, respectively. Due to their significant economic impact and potential for disease transmission, CaPV diseases are listed diseases by the World Organization for Animal Health (OIE).

Sheeppox is characterized by fever, ocular and nasal discharges, papular dermatitis, and nodular lesions in a variety of organs, including the lungs, trachea, and abomasum (6, 16). Young animals are most susceptible, where mortality rates can be as high as 50 to 70% (21, 26). Transmission of sheeppox is thought to require close contact between animals and to occur primarily via aerosol and respiratory droplet, though insect vectors have also been implicated (21, 26, 33, 34). Sheeppox is endemic throughout southwest and central Asia, the Indian subcontinent, and African regions north of the equator (9, 16).

SPPV and other CaPVs are morphologically and serologically indistinguishable and genomically very similar, with overall conservation in genetic content and gene synteny and >96% nucleotide identity (7, 22, 32, 61). Comparison of field and vaccine strains has shown that relatively few genomic changes account for viral attenuation in CaPV. A number of

these genes are predicted to affect virulence and host range (31, 61). Among the genes affected in vaccine strains of CaPV were those with products similar to kelch-like proteins encoded by eukaryotes and other poxviruses.

Kelch-like proteins, including the prototype kelch encoded by *Drosophila*, are members of a protein superfamily characterized by the presence of kelch repeat motifs. These proteins also belong to a subset of proteins characterized by a C-terminal domain of multiple kelch repeats joined to a N-terminal BTB/POZ (for broad-complex, tramtrack and bric-à-brac/poxvirus and zinc finger) domain via a BACK (for BTB and C-terminal kelch) domain (1, 57, 58). Kelch-like proteins are encoded by metazoans and demonstrate considerable expansion in gene repertoire among vertebrates, with humans encoding more than 50 (48). Kelch-like proteins, whose BTB and kelch domains can mediate protein-protein interactions, affect multiple cellular processes, including cytoskeleton organization and dynamics, ion channel modulation, transcriptional regulation, and protein ubiquitination, where they serve as substrate adapter proteins that mediate interaction between ubiquitin ligase complexes and the substrates targeted for degradation (1, 2, 14, 20, 49).

Kelch-like proteins are encoded by members of chordopoxviral genera *Orthopoxvirus*, *Capripoxvirus*, *Leporipoxvirus*, *Suipoxvirus*, and *Yatapoxvirus* and by unassigned deerpox viruses. Poxviral kelch-like genes are generally present as multiple, divergent, and occasionally fragmented genes located in variable and nonessential regions near genomic termini, leading to the suggestion that they function in aspects of virus-host interactions (55). Although domain structure is maintained, amino acid identity between poxviral and cellular kelch-like proteins

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is low. Studies examining the role of poxviral kelch-like genes in virus replication and pathogenesis have been limited to orthopoxviruses, where deletion of single or multiple kelch-like genes have been shown to affect lesion size, inflammatory cell infiltration, or virulence in a mouse model or vascularization and virus yield in a chorioallantoic membrane model of infection (4, 36, 47). These results have suggested a role for kelch-like genes in poxvirus virulence and host range, perhaps through the modulation of inflammatory responses (47). Still, the role of individual kelch-like proteins in poxvirus virulence remains to be fully elucidated.

SPPV encodes three kelch-like proteins—SPPV-019, SPPV-144, and SPPV-151 (annotated as SPPV_16, SPPV_137, and SPPV_144, respectively, in NCBI Reference Sequence accession NC_004002)—that exhibit 93 to 95% amino acid identity to GTPV and LSDV orthologues (61). Examination of *SPPV-019* orthologues in GTPV G20 and LSDV Neethling vaccine strains shows truncating frameshift mutations at positions 231 and 124, respectively, suggesting a role for CaPV *SPPV-019*-like genes in virus virulence (31, 60). In the present study, we examined the role of the kelch-like protein-encoding gene *SPPV-019* in sheep, the natural SPPV host. Our data indicate that *SPPV-019* markedly affects SPPV virulence after intranasal or intradermal virus inoculation.

MATERIALS AND METHODS

Viruses and cell cultures. The pathogenic field isolate SPPV strain A (SPPV-SA) was obtained from a sick sheep in the Almatinskaya region of Kazakhstan and passed nine times in sheep at the Scientific Research Agricultural Institute, Kazakhstan (61). Primary lamb kidney (LK) cells were obtained from the Animal and Plant Health Inspection Service, U.S. Department of Agriculture, at the Plum Island Animal Disease Center, Greenport, NY, and grown in Dulbecco modified Eagle medium containing 10% fetal bovine serum and 50 μ g of gentamicin/ml.

Construction of SPPV recombinant viruses Δ TK, Δ KLP, and its revertant RvKLP. Recombinant SPPVs lacking the *SPPV-019* kelch-like gene or the *SPPV-066* thymidine kinase gene (Δ KLP and Δ TK, respectively), a gene known to be nonessential for capripoxvirus replication, were constructed (51, 63). Recombinant SPPVs were generated by homologous recombination between parental SPPV-SA and recombination transfer vectors containing appropriate gene deletions after viral infection (multiplicity of infection [MOI] of 10) and vector transfection of LK cells (Lipofectamine 2000; Invitrogen, San Diego, CA) (65). For vector construction, a cassette containing the *Escherichia coli* β -glucuronidase (GUS) gene downstream of a synthetic vaccinia virus (VACV) promoter (VVp) (11) and flanked by viral sequences was PCR assembled according to methods previously described (62). Briefly, VVp-GUS and 1 kbp each of *SPPV-019* or *SPPV-066* flanking sequences were PCR amplified by using primers that generate DNA fragments with overlapping ends. The fragments were sequentially fused by recombinant PCR to generate the complete cassette, which was cloned into the pCR2.1 vector (Invitrogen, San Diego, CA). Primers included the following (5' to 3'): GUS forward, TCCCTTATGTTA CGTCTGTAG; GUS reverse, TTCATTGTTTGCTCCCTG; VVp forward, CTAGCGATCGCGTTGCTGAAAAATTGAAATTTATTTTTTTT TTTTGG; VVp reverse, CTTTAAAAATAAAAAAACCCTTATATTT ATACATGTCAGGACATC; *SPPV-019* left flank forward (S19Lf), CTAAA TCATCATCAACAACCTTCATTG; *SPPV-019* left flank reverse (S19Lr), CA GCAACGCGATCGCTACGTTATAGGAGCATTAGACATAGATAGTG; *SPPV-019* right flank forward (S19Rf) CTAAGCGGCCGCGTAAGTATTC AAGATAATTAACAAAAAGTTG; *SPPV-019* right flank reverse (S19Rr), TAGATGATACGTATACGTGTGTGG; *SPPV-066* left flank forward (S66Lf), TTTATTTTACTCCTGTATTATAGAACCTAC; *SPPV-066* left flank reverse (S66Lr), CAGCAACGCGATCGCTATAGGTCCTATAATTA AATGTATATATCCATAG; *SPPV-066* right flank forward (S66Rf), CTAA GCGGCCGCGTAAGGCACTAGATAGCACATTTCAACG; and *SPPV-066* right flank reverse (S66Rr), TGTGTGTCGTCATCTATTAATATCC. Primer pairs S19Lf/S19Rr and S66Lf/S66Rr were used to generate full-length transfer cassettes. Primer pairs S19Tf (CTATGTCTAATGCTCCTATAACG

TTC)/S19Tr (TGTATAGTTAATAAAGTTGAAAGTTGGTC) and S66Tf (ATGGACTATGGAT ATATACATTTAATTATAGTTCTA)/S66Tr (AAA AATAACATTTCTACAAAC) were used to test for the presence of *SPPV-019* and *SPPV-066*, respectively.

LK cell cultures were infected with harvested cell lysates from the infection-transfection and overlaid with complete medium containing 0.5% SeaKem GTG agarose (Cambrex Bioscience, Rockland, ME) and 5-bromo-4-chloro-3-indolyl β -D-glucuronic acid (X-Gluc). Blue plaques indicative of recombinant viruses were collected and purified to homogeneity by additional plaque assays. Lack of *SPPV-019* or *SPPV-066* gene sequences and the correct position and orientation of reporter sequences in recombinant viruses were confirmed by PCR analysis. A 4-kbp genomic region flanking the deletion site was sequenced by using an Applied Biosystems Prism 3730xl automated DNA sequencer (Applied Biosystems, Foster City, CA).

A *SPPV-019* revertant virus (RvKLP) was constructed by restoring *SPPV-019* gene sequences to the Δ KLP genome. A region of the SPPV-SA genome, including the *SPPV-019* gene and flanking regions, was PCR amplified using primers S19Lf and S19Rr and cloned into pCR2.1. Isolated clones were sequenced using overlapping gene-specific primers. Transfer vectors with the correct SPPV sequences were used for RvKLP construction. In addition, a second transfer vector, containing a single nucleotide insertion that introduced a stop codon (amino acid 231) in the linker region between BTB/BACK and kelch-repeat domains of SPPV-019, was used to create a second revertant virus, fsKLP. LK cells were transfected with these transfer vectors and infected with Δ KLP. Harvested viruses were used to infect LK cells in 96-well plates, and supernatants were assayed for the restoration of wild-type *SPPV-019* sequences by dot blot hybridization (protocol adapted from the method of Goswami and Glazer [24]) on a nylon membrane (Hoffmann-La Roche, Switzerland) using a *SPPV-019*-specific digoxigenin-labeled probe generated with primer pair S19Tf/S19Tr (PCR DIG probe synthesis kit; Hoffmann-La Roche). Positive wells were detected using a DIG luminescence detection kit (Hoffmann-La Roche). RvKLP and fsKLP virus clones were purified through two additional rounds of infection and hybridization. A 5-kbp region extending 0.5 kbp outside of either end of the original transfer cassette was then sequenced to ensure the integrity of *SPPV-019* and adjacent genomic regions and to confirm the fsKLP sequence.

Ca²⁺-independent cell adhesion assay. Ca²⁺-independent cell adhesion was assessed as previously described (47, 54), using the divalent cation chelator EDTA instead of EGTA. LK cells grown on chamber slides (Nunc, Inc., Naperville, IL) were either mock infected or infected with SPPV-SA, Δ KLP, or RvKLP viruses (MOI of 10) and allowed to incubate for 24 h. Cells were then washed with phosphate-buffered saline (PBS), treated with 0.1 mM EDTA, and monitored at 5-min intervals for cell adhesion using an inverted microscope and an attached Nikon CoolPix 5000 digital camera. The results were obtained from three independent experiments, and the percent rounded cells was calculated from three randomly selected fields per time point.

Animal inoculation. Three- to four-month-old Merino lambs were inoculated intranasally with 10⁶ PFU of the SPPV-SA, Δ KLP, Δ TK, or RvKLP viruses/animal. Clinical signs of sheepox, including fever (a rectal temperature equal to or greater than 39.7°C), anorexia, lethargy, nasal and ocular discharges, coughing, labored respiration, recumbency, and the appearance of skin lesions, were monitored daily. Blood samples and nasal swabs were collected every day for the first 10 days and every other day thereafter for the duration of the 30-day experiment. Lambs were euthanized when found moribund or at the end of the experiment, and tissue samples were collected. Tissue samples were immediately frozen at -70°C for virus isolation and titration or fixed in 10% neutral buffered formalin for histopathology. Frozen tissues were weighed, homogenized and clarified by low-speed centrifugation. Virus isolation from clinical samples was performed with LK cells as described in OIE protocols (http://www.oie.int/eng/normes/mmanual/A_00033.htm). Virus titration in buffy coat and tissue homogenates was performed by the limiting-dilution method. Titers were calculated by the method of Spearman-Kärber and are expressed as the 50% tissue culture infectious dose (TCID₅₀) (18).

Additional lambs were intradermally inoculated in the left flank and inner side of the left thigh with 10⁴ PFU of SPPV-SA, Δ KLP, or fsKLP virus or mock infected with PBS. Lambs were monitored and sampled as described above and then euthanized and necropsied as described above at 6 days postinfection or at the conclusion of the 10-day experiment.

Real-time PCR. Real-time PCR was used to detect the presence of viral DNA in blood samples. DNA was extracted from buffy coats by using a QIAamp DNA blood minikit (QIAGEN, Inc., Valencia, CA). Reaction mixes were prepared and run with reagents from a SYBR green PCR master mix kit (Applied Biosystems) as recommended in a final volume of 25 μ l. The forward and reverse primers were 5'-GCCAAAAAATTACCATATCAAGGTC-3' and 5'-GAGC

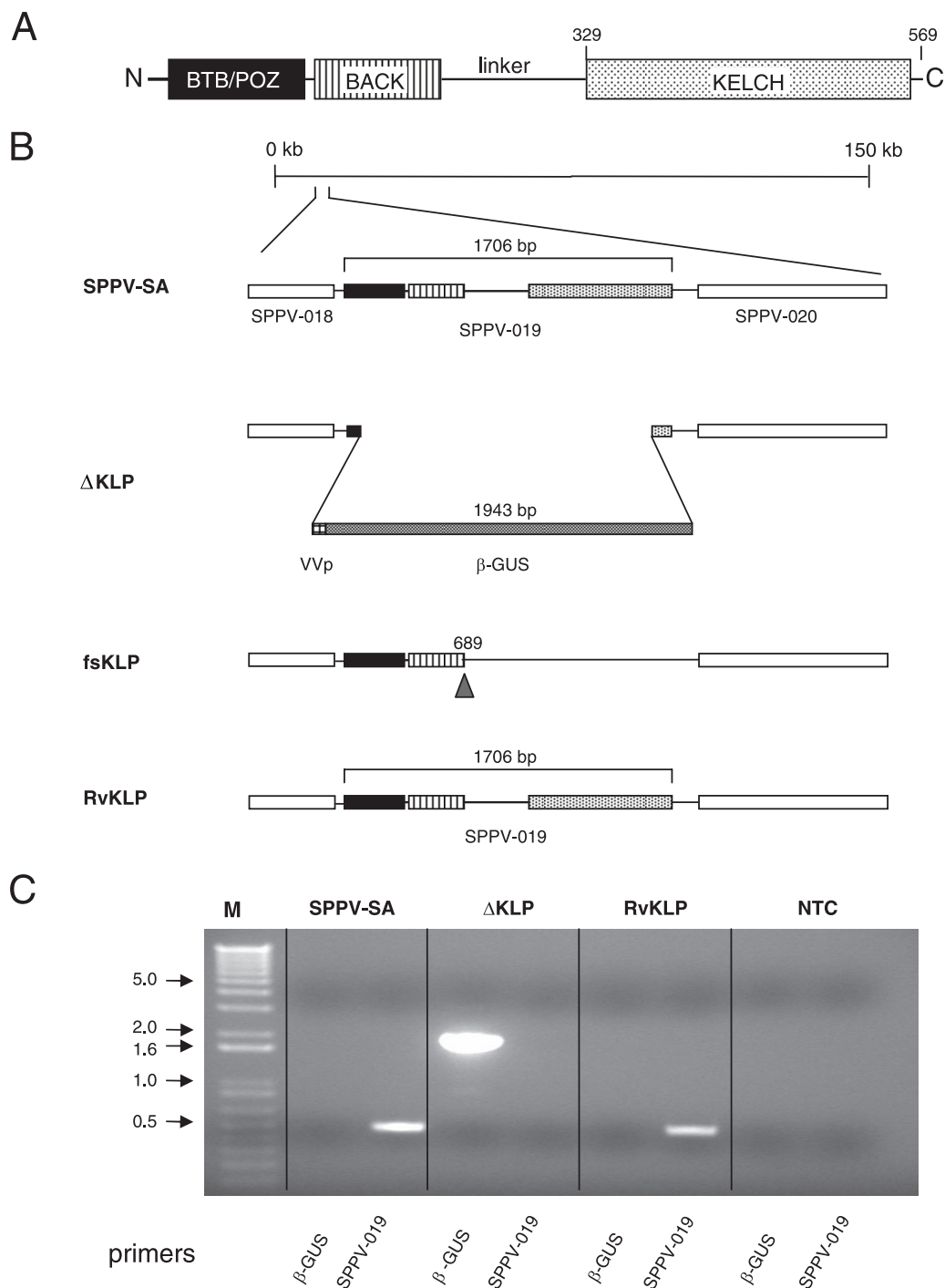


FIG. 1. Characterization of a SPPV *SPPV-019* gene deletion mutant, Δ KLP, and its revertant, RvKLP. (A) Diagram of SPPV-019 domain structure. (B) Diagram of SPPV-SA and recombinant viruses Δ KLP, fsKLP, and RvKLP. The arrowhead in fsKLP indicates the position of the stop codon. (C) PCR analysis of SPPV-SA, Δ KLP, and RvKLP viral DNAs using primers specific for *SPPV-019* or β -glucuronidase (β -GUS) gene sequences. M, molecular markers; NTC, no template DNA.

TGATCCAACATATACTATTGTGC-3' (500 nM), respectively. Sample template (2.5 μ l) was added to each reaction in a SmartCycler 25- μ l reaction tube (Cepheid, Sunnyvale, CA). Cycling conditions consisted of an initial denaturation at 95 $^{\circ}$ C for 5 min, followed by an additional 45 amplification cycles (95 $^{\circ}$ C for 15 s, 58 $^{\circ}$ C for 15 s, and 68 $^{\circ}$ C for 60 s). Assays were run on a Cepheid SmartCycler. Positive and negative controls were included with each set of reactions.

Immunohistochemistry. Tissues were fixed in 10% neutral buffered formalin for 48 h, embedded in paraffin, and sectioned onto SuperFrost Plus glass slides (Fisher Scientific, Pittsburgh, PA). Sections were deparaffinized and rehydrated according to standard methods, unmasked with Dako antigen retrieval solution (DakoCytomation, Carpinteria, CA), and blocked with 2% normal horse serum in PBS. Sections were sequentially incubated with polyclonal anti-SPPV serum (1:10) overnight, biotinylated universal antibody (Vector Laboratories, Burlin-

game, CA) for 1 h, ABC-AP (Vector Laboratories) for 30 min, and Vector Red substrate solution (Vector Laboratories) for an additional 30 min. The slides were counterstained with Mayer's hematoxylin (Sigma-Aldrich, St. Louis, MO), dehydrated, cleared, and mounted.

RESULTS

***SPPV-019* encodes a kelch-like protein.** The predicted 569-amino-acid SPPV-019 protein is similar to kelch-like proteins encoded by chordopoxviruses and metazoans (48). Present in SPPV-019 are the domains conserved in other kelch-like proteins, including an N-terminal BTB/POZ domain (Pfam 00651, amino acids 15 to 116), a BACK domain (Pfam 07707, amino acids 121 to 220), and a C-terminal kelch domain (amino acids 329 to 564) containing four kelch repeat motifs (Pfam 01344) (Fig. 1A). The four intact kelch repeats are preceded by one repeat that is incomplete and lacks the conserved tryptophan residue of the β 4 sheet within the predicted kelch domain propeller blade structure (1). The BTB-BACK and the kelch-repeat-containing domains are separated by a 109-amino-acid linker region.

SPPV-019 is most similar to its orthologues in other chordopoxviral genera, sharing 35 to 40% amino acid identity to orthologues in genera closely related to CaPV (suipoxvirus, yatapoxviruses, leporipoxviruses, and deerpox viruses) and 24 to 27% identity to orthopoxviral orthologues, including VACV (strain Copenhagen) F3L. The orthologous nature of these genes is further indicated by the conserved locus at which they are located in all chordopoxviral genomes containing kelch-like genes. This locus is in the left genomic region between the dUTPase gene (orthopoxviruses, capripoxviruses, yatapoxviruses, and deerpox viruses) or myxoma virus *m013L*-like gene (leporipoxviruses and suipoxvirus) and the ribonucleotide reductase small subunit gene (61). Notably, *SPPV-019* orthologues represent the only kelch-like gene present in all poxviruses known to encode kelch-like proteins, making it the most conserved poxviral kelch-like gene. Sequence conservation among SPPV-019 orthologues is lowest in the linker region separating the BACK domain from the kelch domain, a region that contains a unique 40-amino-acid sequence in CaPV orthologues. SPPV-019 is less similar to paralogues in SPPV (18 to 24% amino acid identity to SPPV-144 and SPPV-151 over their length), homologues in other poxvirus genera (including VACV A55R and C2L [23 and 24% amino acid identity, respectively]), and cellular kelch-like proteins.

Construction and analysis of SPPV *SPPV-019* and *SPPV-066* gene deletion mutants, Δ KLP and Δ TK, and the *SPPV-019* revertant, RvKLP. To examine the role of *SPPV-019* during SPPV infection, an SPPV *SPPV-019* gene deletion mutant, Δ KLP, and its revertant, RvKLP, were constructed. In Δ KLP, 1,654 bp of the 1,706-bp *SPPV-019* gene (98.5%) were deleted and replaced with the 1,943-bp reporter gene cassette. In RvKLP, Δ KLP reporter gene sequences were replaced with wild-type *SPPV-019* sequences (Fig. 1B). An additional revertant virus, fsKLP, containing a nonsense mutation at SPPV-019 amino acid position 231 was also constructed (Fig. 1B). As a control for the generation of gene deletion mutants, an *SPPV-066* thymidine kinase gene deletion mutant (Δ TK) was constructed by replacing the 5' 307 bp of the *SPPV-066* coding sequence (531 bp) with the GUS cassette. Genomic DNA from

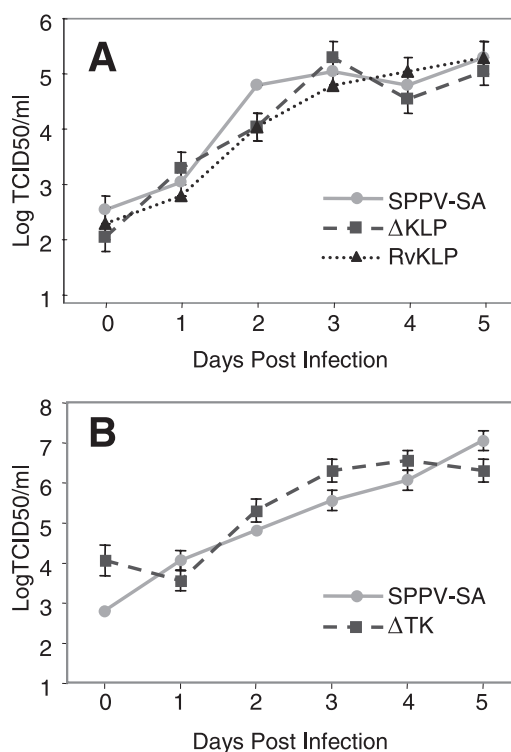


FIG. 2. Growth characteristics of SPPV isolate SPPV-SA and recombinant SPPV viruses Δ KLP, RvKLP (A), and Δ TK (B) in primary sheep LK cells. Cells were infected (MOI = 0.1) with the appropriate viruses and, at the indicated times postinfection, samples were collected and titrated for virus yield. The data are the means and standard errors of three independent experiments.

parental and recombinant viruses was analyzed by PCR. The deleted *SPPV-019* sequences were not detected in DNA preparations prepared from Δ KLP virus stocks but were amplified from parental and revertant viral genomic DNAs. Reporter gene sequences were detected only in the deletion mutant virus (Fig. 1C). Similar results were obtained for Δ TK (not shown). DNA sequencing confirmed preservation of wild-type genomic sequences flanking the deletion sites in recombinant viruses and integrity of the wild-type *SPPV-019* gene in RvKLP (not shown).

***SPPV-019* does not affect growth of SPPV in vitro.** Growth characteristics of Δ KLP were compared to those of SPPV-SA and RvKLP by infecting primary LK cell cultures (MOI = 0.1) and determining total virus titers at 24-h intervals for 5 days. No significant differences in growth were observed between these viruses, indicating that *SPPV-019* is not essential for SPPV growth in LK cells (Fig. 2A). As anticipated, the growth characteristics of Δ TK also were indistinguishable from those of SPPV-SA (Fig. 2B). In addition, no differences in plaque size or morphology were observed for Δ KLP relative to the parental virus (not shown).

***SPPV-019* affects Ca^{2+} -independent cell adhesion in LK cells.** Of interest was the ability of Δ KLP to affect Ca^{2+} -independent cell adhesion in infected cultured cells, a phenotype previously shown to be affected in VACV-infected cells by deletion of C2L and A55R kelch-like genes (4, 47, 54). To examine the ability of SPPV to affect cell adhesion in vitro and

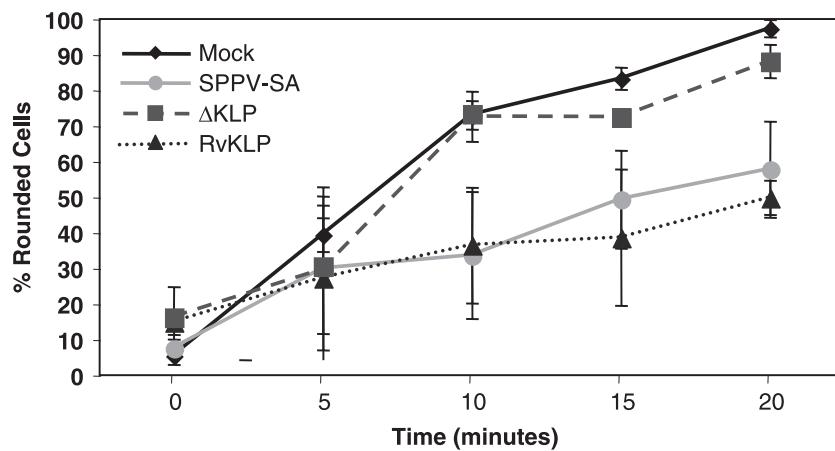


FIG. 3. Ca^{2+} -independent adhesion of LK cells infected with SPPV-SA, Δ KLP, or RvKLP viruses. Monolayers of LK cells were infected (MOI = 10) with the indicated viruses or mock infected. At 24 hpi, cultures were treated with EDTA and photographs of cell monolayers under phase-contrast microscopy were taken at 5-min intervals. The data were collected from three independent experiments, and the percent rounded cells was calculated as described in Materials and Methods. The number \pm the standard deviation of rounded cells is shown.

the effect of *SPPV-019* gene deletion on this phenotype, LK cells were mock infected or infected with Δ KLP, SPPV-SA, or RvKLP for 24 h, followed by treatment of cultures with EDTA (Fig. 3). Although 97% of mock-infected cells were rounded and detached from the glass surface within 20 min of EDTA treatment, ca. 50% of cells infected with SPPV-SA or RvKLP remained adherent. This indicates that SPPV infection induces Ca^{2+} -independent adhesion in LK cells. Cells infected with Δ KLP displayed a phenotype intermediate (88% of cells rounded and detached) relative to SPPV-SA or RvKLP and mock-infected cells, indicating that *SPPV-019* contributes directly or indirectly to the Ca^{2+} -independent adhesion phenotype in LK cells.

***SPPV-019* affects viral virulence in sheep.** (i) **Intranasal inoculation.** To examine the role of *SPPV-019* on SPPV virulence, Merino lambs were inoculated intranasally with 10^6 TCID₅₀ of SPPV-SA, Δ KLP, or Δ TK (experiment 1) or SPPV-SA, Δ KLP, or RvKLP (experiment 2) and observed for clinical signs of infection (Tables 1 and 2). In contrast to SPPV-SA and RvKLP, where the combined mortality was 92%, all lambs infected with Δ KLP and Δ TK survived the infection (Table 1). Although lambs in all groups showed a fever response starting

on days 3 to 4 postinoculation, the mean body temperatures were highest in lambs inoculated with SPPV-SA or RvKLP ($>40.2^\circ\text{C}$), lowest in the Δ TK group (39.3°C), and intermediate in Δ KLP-infected lambs (39.7°C). Pyrexia persisted until death in the SPPV-SA and RvKLP groups but ended after approximately 1 or 2 weeks in Δ TK and Δ KLP groups, respectively. All lambs inoculated with SPPV-SA (8 of 8 animals) or RvKLP (5 of 5 animals) exhibited seromucous or purulent nasal discharge. In contrast, only one Δ KLP-infected lamb and no Δ TK-infected lambs exhibited nasal discharge (Table 1).

Differences in the development of skin lesions were observed between experimental groups. Lambs inoculated with SPPV-SA or RvKLP exhibited multiple skin lesions, which appeared first in axillary, inguinal, and perineal regions and eventually covered the entire body of most animals (Table 1). Skin lesions progressed from macular to papular to nodular forms (Fig. 4A). In contrast, of the eight lambs inoculated with Δ KLP, three failed to develop skin lesions, four exhibited a few (<6) transient prepapular axillary and/or inguinal lesions (Fig. 4B), and only one animal exhibited papular skin lesions (approximately 15) (Table 1). No skin lesions were observed in lambs inoculated with Δ TK.

TABLE 1. Survival and clinical response after intranasal inoculation of lambs with SPPV-SA, Δ TK, Δ KLP, or RvKLP viruses^a

Expt and virus	Survival		Fever			Nasal discharge (no. of lambs/ total no.)	Skin lesions	
	No. of lambs surviving/ total no.	Mean days to death \pm SEM	Mean days to onset \pm SEM (no. of lambs with fever)	Duration (mean no. of days \pm SEM)	Mean temp ($^{\circ}$ C) \pm SEM		No. of lambs with lesions/ total no.	Mean days to onset \pm SEM
Expt 1								
SPPV-SA	0/5	16.4 \pm 3.3	3.6 \pm 1.8 (5)	13.6 \pm 5.4	40.5 \pm 0.8	5/5	5/5	6.8 \pm 1.1
Δ KLP	5/5	NA	4.6 \pm 1.9 (4)	13.0 \pm 1.2	39.7 \pm 0.5	0/5	4/5	6.0 \pm 1.6
Δ TK	5/5	NA	3.6 \pm 2.2 (5)	6.6 \pm 3.4	39.4 \pm 0.4	0/5	0/5	NA
Expt 2								
SPPV-SA	1/3	17.5	4.0 \pm 1.0 (3)	18.7 \pm 6.0	40.3 \pm 0.7	3/3	3/3	6.7 \pm 0.6
RvKLP	0/5	17.4 \pm 3.0	4.4 \pm 0.5 (5)	13.4 \pm 3.0	40.4 \pm 0.7	2/5	5/5	5.6 \pm 0.5
Δ KLP	3/3	NA	4.3 \pm 0.6 (3)	15.7 \pm 2.5	39.7 \pm 0.7	1/3	1/3	7

^a Lambs were inoculated with 10^6 TCID₅₀ of the indicated viruses and examined for clinical signs of infection for 30 days. NA, not applicable.

TABLE 2. Detection of SPPV in nasal secretions and blood after intranasal inoculation of lambs with SPPV-SA, Δ KLP, Δ TK, or RvKLP virus^a

Expt and virus	Nasal secretions			Viremia		
	No. of lambs shedding virus/total no. inoculated	Mean days to onset postinoculation \pm SEM	Duration (mean no. of days \pm SEM)	Mean days to onset \pm SEM (no. of viremic lambs)	Duration (mean no. of days \pm SEM)	Maximum viremia (\log_{10} TCID ₅₀ /ml) \pm SEM
Expt 1						
SPPV-SA	5/5	8.4 \pm 1.7	8.0 \pm 2.0	5.6 \pm 0.9 (5)	10.8 \pm 3.0	ND
Δ KLP	0/5	NA	NA	7.0 \pm 2.0 (4)	3.5 \pm 1.0	ND
Δ TK	0/5	NA	NA	ND	ND	ND
Expt 2						
SPPV-SA	3/3	3.7 \pm 0.6	11.0 \pm 1.0	4.0 \pm 0.0 (3)	7.7 \pm 0.6	4.7 \pm 0.5
RvKLP	5/5	4.0 \pm 0.0	10.0 \pm 1.4	3.8 \pm 0.4 (5)	7.8 \pm 0.4	4.2 \pm 0.9
Δ KLP	2/3	10.5	6.5	5.7 \pm 1.5 (3)	5.7 \pm 1.5	2.8 \pm 0.5

^a Lambs were inoculated with 10^6 TCID₅₀ of the indicated viruses. Virus detection in secretions and virus titers in buffy coats was performed with LK cell cultures. Virus detection in buffy coats was performed using real-time PCR. NA, not applicable; ND, not done.

Although all lambs inoculated with SPPV-SA or RvKLP and seven of eight lambs inoculated with Δ KLP became viremic, the appearance of Δ KLP in blood (real-time PCR) was delayed approximately 1.5 days relative to SPPV-SA or RvKLP. Further, the duration of viremia and maximum viremic titers in animals infected with Δ KLP were reduced (Table 2). No virus was detected in blood collected from the Δ TK group. Infectious virus was detected in nasal secretions from all animals infected with SPPV-SA or RvKLP. In contrast, only two of eight lambs inoculated with Δ KLP shed virus in nasal secretions, and the onset of virus shedding was delayed and transient relative to the SPPV-SA or RvKLP groups (Table 2). Lambs in the Δ TK group did not shed detectable virus in nasal secretions.

Upon postmortem examination, lambs inoculated with SPPV-SA or RvKLP demonstrated tracheal ulcers (10 of 13 animals) and lesions in the rumen (6 of 13 animals), lungs (6 of 13 animals), and nasal turbinates (2 of 13 animals). In contrast, lambs inoculated with Δ KLP or Δ TK lacked postmortem lesions, with the exception of a single tracheal ulcer in one Δ KLP-infected lamb. These results demonstrated that deletion of *SPPV-019* markedly attenuated SPPV in sheep after intranasal inoculation.

(ii) **Intradermal inoculation.** To determine whether *SPPV-019*-dependent attenuation is affected by inoculation route, two groups of three lambs were intradermally inoculated in the flank and in the inner side of the left thigh with 10^4 TCID₅₀ of

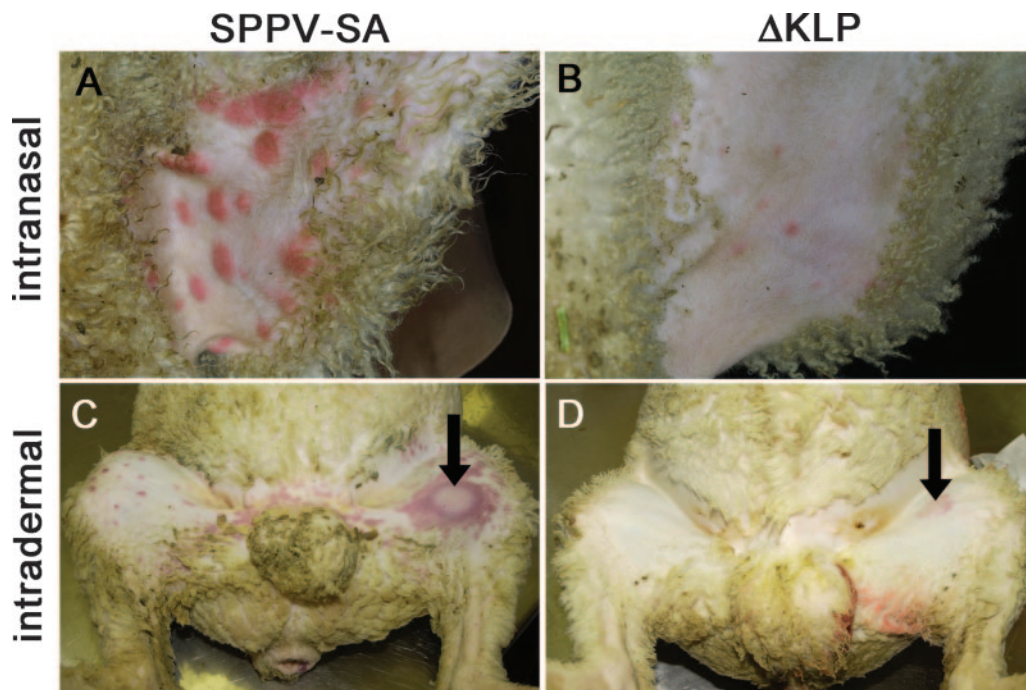


FIG. 4. Skin lesions in lambs 8 days after intranasal (A and B) or intradermal (C and D) inoculation with SPPV-SA (A and C) or Δ KLP (B and D) viruses. Animals were inoculated with 10^6 (intranasal) or 10^4 (intradermal) TCID₅₀. Panels A and B show the left axillary regions. In panels C and D, arrows indicate inoculation sites in the inner side of the left thigh.

TABLE 3. Clinical signs, lesions at necropsy, and virus titers after intradermal inoculation of lambs with SPPV-SA or Δ KLP viruses^a

Parameter	6 dpi		10 dpi			
	SPPV-SA (animal 14)	Δ KLP (animal 20)	SPPV-SA		Δ KLP	
			Animal 15	Animal 17	Animal 18	Animal 19
Clinical						
Lesion size at inoculation site ^b (mm)						
Flank	32	29	53	30	40	33
Thigh	60	30.5	47	47	0	32
Maximum temp (°C) (no. of days)	39.4 (1)	39.7 (1)	40.8 (7–10)	40.8 (5, 9)	40.1 (7–9)	39.8 (7)
NIS ^c skin lesions (days)	Papula (5–6)	No lesions	Papula (5–10)	Papula (5–10)	No lesions	Prepapula (6–8)
Virus shedding ^d (day)	Negative	Negative	Positive (10)	Positive (10)	Negative	Negative
Postmortem lesions						
NIS ^c skin	Generalized	None	Generalized	Generalized	None	None
Lung	Multiple	None	Multiple	Multiple	None	Few
Rumen	Multiple	None	Multiple	Multiple	None	Few
Virus titer^e (mean \pm SEM)						
Inoculation site						
Flank	4.8 \pm 0.0	3.5 \pm 0.3	7.8 \pm 0.0	4.8 \pm 0.5	6.8 \pm 0.5	5.8 \pm 0.0
Thigh	7.4 \pm 0.3	5.8 \pm 0.0	6.5 \pm 0.3	7.5 \pm 0.3	0	5.8 \pm 0.0
Perilumbar LN	4.8 \pm 0.0	0	4.5 \pm 0.5	4.1 \pm 0.3	0	0
Inguinal LN	6.1 \pm 0.3	0	3.8 \pm 0.0	3.8 \pm 0.0	0	0
Prescapular LN	NA	NA	3.1 \pm 0.3	3.1 \pm 0.3	0	0
Lung	NA	NA	0	0	0	0
Viremia ^f (days detected) (<i>C_T</i>)	4–6 (29.5)	6 (42.5)	4–10 (30.4)	4–10 (28.3)	8 (41.6)	8–10 (32.2)

^a Lambs were inoculated with 10⁴ TCID₅₀ SPPV-SA or Δ KLP or mock infected with PBS and then sacrificed at 6 or 10 days postinoculation (dpi).^b At time of necropsy.^c NIS, noninoculation site.^d Buccal swabs were tested for the presence of virus by virus isolation.^e Presented as the TCID₅₀/mg of tissue \pm SEM. LN, lymph node.^f That is, the viral genomic DNA in buffy coats as detected by real-time PCR, including the average threshold cycle (*C_T*) in parentheses.

SPPV-SA or Δ KLP and euthanized and necropsied at either 6 or 10 days postinoculation (Table 3). All inoculated lambs developed large, raised, erythematous lesions at the inoculation sites, although one lamb in the Δ KLP group developed a lesion in the flank but not in the thigh. Although lesion size at thigh inoculation sites tended to be larger in SPPV-SA- than in Δ KLP-infected lambs, differences between groups were not significant. Lambs inoculated with SPPV-SA developed high fever ($>40.5^{\circ}\text{C}$) and generalized skin lesions (Fig. 4C), whereas lambs inoculated with Δ KLP showed mild fever and either no secondary skin lesions (Fig. 4D) or transient pre-papular lesions (Table 3). Detection of viral DNA in blood was delayed by 2 to 4 days in Δ KLP-infected animals relative to those infected with SPPV-SA (Table 3). Lambs infected with SPPV-SA began shedding virus in nasal secretions by day 10, while lambs infected with Δ KLP did not shed detectable virus. Postmortem examination revealed multiple lesions in the lungs and rumina of lambs inoculated with SPPV-SA, whereas only one of the lambs inoculated with Δ KLP showed a few scattered focal lesions in these organs. Tissue samples from the inoculation sites and draining lymph nodes, axillary skin, and lungs were collected for virus titration. Although virus was detected in the skin and lymph nodes from all lambs infected with SPPV-SA, no virus was detected in lambs inoculated with Δ KLP other than at the inoculation sites (Table 3). On day 10, no significant difference in virus titer was observed from skin inoculated with SPPV-SA or Δ KLP (Table 3). Furthermore, histology and immunohistochemistry of skin lesions at inocu-

lation sites exhibited no differences in pathology or viral antigen distribution between groups (not shown). These results indicate that Δ KLP, while replicating at the site of intradermal inoculation, is restricted in its ability to disseminate and cause generalized disease.

As an additional control for the Δ KLP phenotype, fsKLP, a virus maintaining *SPPV-019* coding sequences but harboring a nonsense mutation at SPPV-019 amino acid position 231, was intradermally inoculated into two lambs as described above. Only one of the two lambs showed a transient fever response (40°C) that persisted for 3 days. Similar to Δ KLP, primary lesions at inoculation sites were observed, but secondary skin lesions were absent.

DISCUSSION

We have shown here that an SPPV kelch-like gene, *SPPV-019*, affects viral virulence in sheep. A recombinant virus with an *SPPV-019* deletion, Δ KLP, was markedly attenuated after virus inoculation by the intranasal or intradermal routes (Tables 1, 2, and 3). Attenuation of Δ KLP relative to parental (SPPV-SA) or revertant (RvKLP) viruses was demonstrated by 100% lamb survival, decreased fever response and nasal discharge, marked reduction in number and development of skin and internal lesions, reduced viremia titers and duration of viremia, and reduced virus shedding. The observed attenuation of Δ KLP in the natural SPPV host species and use of the natural route of infection indicates that *SPPV-019* is a bona

fide virulence determinant for SPPV. This represents the first attenuation of virulent SPPV or any other pathogenic CaPV by engineered gene deletion.

The marked decrease in lesion number and the limited progression of lesion development exhibited by lambs intranasally or intradermally inoculated with Δ KLP (Tables 1 and 3) may be partially explained by the reduced magnitude and duration of viremia (Tables 2 and 3). In lambs inoculated by the intradermal route, Δ KLP failed to spread to lymph nodes, and secondary skin and lung lesions were less numerous and less pronounced than in lambs inoculated with wild-type virus (Table 3). However, lesion size and Δ KLP skin titers at dermal inoculation sites were comparable to those of SPPV-SA. Overall, these data suggest that Δ KLP is restricted in its ability to spread to secondary sites of infection.

Growth characteristics of Δ KLP in primary sheep cells (LK) were indistinguishable from those of SPPV-SA or RvKLP (Fig. 2), indicating that *SPPV-019* is not essential for virus growth in these cells and suggesting that, similar to kelch-like genes present in orthopoxviruses, *SPPV-019* is dispensable for growth in vitro (36, 37, 45). In CPXV, deletion of kelch-like genes *D11L*, *G3L*, *C18L*, or *A57R* did not affect virus growth in a number of cell lines; however, simultaneous deletion of three or four of these genes delayed virus replication in certain cell lines, suggesting an additive or gene dosage effect for kelch-like genes in some aspects of host range (36). Whether an additive effect would be seen with double or triple SPPV kelch-like gene deletion mutants remains to be determined.

The present study demonstrates that deletion of a single viral kelch-like gene from SPPV leads to marked attenuation of virus in the natural host. Previous studies deleting single kelch-like genes in CPXV and VACV failed to reduce viral virulence in mice. Further, CPXV double kelch-like gene knockouts in mice caused disease indistinguishable from that of wild-type CPXV, and a significant decrease in virus virulence was observed only with quadruple kelch-like gene deletion mutants (36). VACV with *C2L* or *A55R* gene deletions exhibited no difference in weight loss or mortality relative to parental virus after intranasal inoculation of mice (4, 47). However, in an intradermal mouse model, infection with one of these two viruses with deletions in one of these two genes resulted in lesions that were larger (*A55R* and *C2L*), took longer to heal, and contained more abundant cell infiltrates (*C2L*) than those observed for parental and revertant viruses, suggesting that *C2L* and *A55R* function by limiting the intradermal pathology of VACV, perhaps by modulating inflammatory responses (4, 47).

The range of phenotypes observed among *SPPV-019* single gene and orthopoxviral kelch-like gene deletion studies may be explained by functional differences of specific poxviral kelch-like proteins individually or within the context of the viral genotype, the nature of the host used, or both. Although all encode kelch-like proteins, *SPPV-019*, VACV *C2L*, and VACV *A55R* are distinct genes. The lack of complementation between VACV *C2L* and *A55R* in vitro and the slight phenotypic differences between the *C2L* and *A55R* gene deletion mutants in mice support functional differences in these genes. In addition, SPPV, VACV, and CPXV genomes contain distinct kelch-like genes and overall gene complements that may influence the effect of individual kelch-like proteins. Finally,

the limited host range of SPPV and its virulence in sheep relative to VACV and CPXV in mice suggest that sheepox may be a useful and appropriate natural host model to functionally determine specific roles of virulence and host range determinants in disease (17, 39).

The function(s) of poxviral kelch-like proteins is unknown. Compared to wild-type VACV, infection of cells with VACV *C2L* or *A55R* gene knockouts resulted in altered plaque morphology and a reduction in the formation of cellular projections and Ca^{2+} -independent cell adhesion in vitro, indicating that *C2L* and *A55R* may play a role in promoting Ca^{2+} -independent cell adhesion and cellular projection formation (4, 47, 54). Similar to VACV *C2L* and *A55R* deletion mutants, Δ KLP-infected cells did exhibit a reproducible 40% reduction in Ca^{2+} -independent cell adhesion (Fig. 3); however, no obvious differences in plaque morphology were observed between SPPV-SA and Δ KLP-infected cultures, and no cellular projections were observed in any SPPV-infected cells (not shown). How *SPPV-019* may specifically affect cellular adhesion or whether a cell adhesion function is involved in failure of Δ KLP to spread to secondary sites within the host have yet to be determined.

Poxviral kelch-like proteins conceivably perform a range of functions during infection, potentially through interaction with cytoskeleton or through protein ubiquitination. The effect of *SPPV-019* on cellular adhesion is consistent with roles of cellular kelch-like proteins in cell attachment and cytoskeletal dynamics (1, 27, 29, 49, 56). The cytoskeleton has also been shown to affect critical processes during VACV infection; however, the phenotypes of Δ KLP described here suggest that functions affected by potential SPPV019-cytoskeletal interaction are distinct and perhaps more host range related (10, 13, 28, 40, 53). Kelch-like proteins have also been shown to function as substrate adaptor molecules for Cul3-based E3 ubiquitin ligase complexes (14, 20, 23, 35, 38, 46). Other poxviral genes have been predicted to encode ubiquitin ligase-related proteins, and poxvirus-encoded ubiquitin ligases have been shown to target host proteins and affect viral virulence and host range, thus making attractive an E3 substrate adapter role for virally encoded kelch-like proteins (3, 25, 41, 42). SPPV-019 could also affect cell attachment through an E3-adaptor protein function, since ubiquitination pathways have been shown to affect cell attachment and cytoskeletal dynamics (19, 30, 64). The precise mechanism(s) through which SPPV-019 or other poxviral kelch-like proteins affect virulence requires further investigation.

SPPV-019 orthologues in vaccine strains GTPV G20 and LSDV Neethling contain frameshift mutations at positions 231 and 124, respectively, suggesting that *SPPV-019* orthologues affect CaPV virulence (31, 60). The results here with SPPV further support the hypothesis that mutation of *SPPV-019* orthologues contributes to the attenuated phenotypes of these vaccines. Similar to GTPV G20, the fsKLP mutant generated here harbors a nonsense mutation in the region separating the BTB and BACK domains from the kelch repeat-containing domain. Interestingly, fsKLP also was attenuated after intradermal virus inoculation of lambs, suggesting that a complete SPPV-019 protein is necessary for virus virulence.

Infection with Δ TK did not affect SPPV replication in tissue culture but resulted in an even more attenuated phenotype

than Δ KLP in vivo. Lambs intranasally inoculated with Δ TK did not shed detectable virus in nasal secretions and, with the exception of a transient, low-magnitude fever response, did not develop signs of disease (Tables 1 and 2), indicating that *SPPV-066* is an SPPV virulence factor in sheep. Preliminary studies using the intradermal route of infection showed that, unlike Δ KLP, Δ TK fails to induce a lesion at the inoculation site, suggesting that viral TK is important for virus growth in the skin (not shown). An important role for viral TK in pathogenesis has been previously reported with other large DNA viruses, including VACV (8, 59), African swine fever virus (43), and herpes simplex virus (12). The TK locus has been used as a site of insertion for foreign viral genes during construction of CaPV vaccine expression vectors; however, these recombinants were derived from previously attenuated CaPV, and the effect of TK deletion on viral virulence has not been reported (5, 15, 50–52).

Sheepox is of major economic significance in the developing world, and vaccination is the most important disease control strategy in areas of endemicity. Attenuated live vaccines, obtained through serial passage of SPPV in tissue culture, have been used with variable success. Problems associated with these vaccines include variable levels and duration of protection, lack of vaccine virus characterization, and safety issues (6). Studies of SPPV virulence determinants in the natural host may be useful to better define mechanisms underlying poxviral pathogenesis and for engineering novel vaccines with improved safety and efficacy and of greater utility.

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ADDENDUM

Since completion of the work described here, deletion of the *SPPV-019* orthologue in VACV (*F3L*) has been reported to result in modest but significant reduction in lesion size and alteration of infiltrating immune cell populations in a mouse intradermal model of VACV infection, a finding suggestive of an effect on innate immune responses (18a). Whether a similar effect on immune response contributes to the attenuated phenotype of the *SPPV-019* gene deletion mutant reported here remains to be determined.

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